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Complementation of African cassava mosaic virus *AC2* gene function in a mixed bipartite geminivirus infection

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We have previously demonstrated that African cassava mosaic virus (ACMV) DNAs A and B efficiently complement the systemic spread of tomato golden mosaic virus (TGMV) DNA A when co-agroinoculated onto *Nicotiana benthamiana*. Here, we show that a mixture of an ACMV DNA A *AC2* mutant and DNA B that is normally unable to systemically infect *N. benthamiana* can do so at low frequency when co-agroinoculated with TGMV DNA A. Analysis of viral DNA showed that the *AC2* mutation was retained during infection. The mixture of genomic components was sap transmissible, indicating that systemic infectivity is not specifically attributable to the use of agroinoculation. In the presence of TGMV DNA A, ACMV coat protein as well as the DNA B gene products BV1 and BC1 were detected in systemically infected tissues. The results demonstrate that dysfunctional *AC2* can be complemented *in planta* by its TGMV homologue *AL2*.

The majority of whitefly-transmitted geminiviruses, such as African cassava mosaic virus (ACMV) (Stanley, 1983; Stanley & Gay, 1983) and tomato golden mosaic virus (TGMV) (Hamilton *et al.*, 1984), have bipartite genomes (DNAs A and B). ACMV DNA A encodes the coat protein (Townsend *et al.*, 1985), gene *AC1* required for viral DNA replication, and the regulatory genes *AC2* and *AC3* (Townsend *et al.*, 1986; Etessami *et al.*, 1991). DNA A alone is able to produce virus particles (Klinkenberg & Stanley, 1990) but DNA B genes *BV1* and *BC1* are essential for efficient virus spread and symptom induction (Etessami *et al.*, 1988).

Disruption of *AC2* expression prevents systemic infection of *Nicotiana benthamiana* although *AC2* mutants retain the ability to replicate in *N. tabacum* protoplasts (Etessami *et al.*, 1988). Studies in which the TGMV coat protein coding sequences have been replaced by the reporter genes β-glucuronidase (GUS) and neomycin phosphotransferase have indicated that coat protein expression is dependent on the activity of *AL2* (the TGMV homologue of ACMV *AC2*) (Sunter *et al.*, 1990; Sunter & Bisaro, 1991; Gröning *et al.*, 1994). The product of this gene acts as a transcription factor for the expression of the coat protein gene and *BR1* (Sunter & Bisaro, 1992), which are located in similar positions on their respective genomic components. Transient expression studies using GUS under the control of ACMV promoters have suggested that the product of *AC2* is additionally required for the expression of *BC1* (Haley *et al.*, 1992).

The production of viable pseudorecombinants by reassortment of genomic components of bipartite geminiviruses is usually confined to closely related strains (Stanley *et al.*, 1985; Lazarowitz, 1991; von Arnim & Stanley, 1992*a*; Frischmuth *et al.*, 1993) because of the highly specific nature of the interaction of AC1 with the origin of viral DNA replication (Fontes *et al.*, 1992; Lazarowitz *et al.*, 1992; Gilbertson *et al.*, 1993). This precludes the use of pseudorecombinants for the study of gene complementation between distinct viruses. However, experiments in which mixtures of genomic components were introduced into *N. benthamiana* have shown that TGMV DNA A spreads efficiently in the presence of ACMV DNAs A and B (Frischmuth *et al.*, 1993), demonstrating that ACMV DNA B genes can functionally interact with the TGMV genomic component. Here, we assess the feasibility of using this type of approach to investigate DNA A gene complementation. Because ACMV *AC2* is essential for infectivity, we have tested the ability of TGMV AL2 to complement dysfunctional AC2 simply by screening for the development of systemic symptoms in *N. benthamiana*.

The construction of pBin19 clones containing either partial or tandem repeats of ACMV DNA A (AA) and DNA B (AB) and common strain TGMV DNA A (TA) and DNA B (TB) in *Agrobacterium tumefaciens* strain LBA4404 has been described (von Arnim & Stanley, 1992*a*; Klinkenberg *et al.*, 1989). Nucleotide numbering of ACMV and TGMV is according to Stanley & Gay

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Table 1. *Infectivity of combinations of ACMV and TGMV genomic components in N. benthamiana*

Inoculum	Experiment*						
	I	II	III	IV	V	VI	VII
AA+AB	6/11	—	2/3	10/10	4/4	4/4	—
AA+AB+TA	9/11	10/15	—	—	3/4	3/5	—
TA+TB	11/11	5/5	3/3	—	4/4	4/4	—
AA-AC2-2+AB	0/11	0/5	—	—	0/4	0/4	0/50
AA-AC2-2+AB+TA	1/11	0/15	1/24	2/20	1/15	1/25	1/50
TA-AR1-1+TB	—	—	—	—	4/4	4/4	—
AA-AC2-2+AB+TA-AR1-1	—	—	—	—	0/15	1/50	—

* Virus genomic components were introduced either by agroinoculation (experiments I, II, III, V, VI and VII) or by sap transmission using infected plants from experiment III (experiment IV).

(1983) and Hamilton *et al.* (1984), respectively. The ACMV mutant AC2-2, containing a *Hpa*I(1651) site and a premature termination codon within the *AC2* coding sequence (Etessami *et al.*, 1991), was cloned as a partial repeat in pBin19 as described for ACMV DNA A (Klinkenberg *et al.*, 1989) to produce AA-AC2-2. A TGMV coat protein mutant was made by digestion of clone csTA (von Arnim & Stanley, 1992*a*) at the unique *Xho*I(400) site, infill of protruding 5′ termini and religation to create an additional *Pvu*I site. Partial repeats of this clone were constructed in pBin19 as described for TGMV DNA A (von Arnim & Stanley, 1992*a*) to produce TA-AR1-1. AA-AC2-2 and TA-AR1-1 were mobilized into *A. tumefaciens* strain LBA4404 by triparental mating (Hoekema *et al.*, 1983; Ditta *et al.*, 1980). ACMV and TGMV were held and manipulated under MAFF licence numbers PHF 1185A/68(21) and PHF 1185B/17(111) under the Plant Pests (Great Britain) Order 1980.

N. benthamiana was maintained in accordance with the requirements of the Advisory Committee on Genetic Manipulation, in an insect-free glasshouse at 25 °C with supplementary lighting to give a 16 h photoperiod. Combinations of ACMV and TGMV genomic components were introduced into plants by stem agroinoculation (Klinkenberg & Stanley, 1990). As expected, both ACMV (AA+AB and AA+AB+TA) and TGMV (TA+TB) were highly infectious and induced severe leaf curl and chlorotic symptoms typical of these viruses in this particular host (Table 1). In contrast, the ACMV *AC2* mutant (AA-AC2-2+AB) was unable to systemically infect *N. benthamiana*, consistent with earlier results when the mutant was introduced as a linearized clone insert by mechanical inoculation (Etessami *et al.*, 1991). However, when the ACMV *AC2* mutant was co-agroinoculated with TGMV DNA A (AA-AC2-2+AB +TA), mild systemic symptoms consisting of chlorosis around the main mid-rib and occasionally around the minor veins of leaves were induced at low frequency (five plants out of 140, over five experiments), suggesting that the ACMV mutant could be complemented by TGMV DNA A. In addition, when the *AC2* mutant was co-agroinoculated with a TGMV coat protein mutant (AA-AC2-2+AB+TA-AR1-1) a single plant (out of 65) showed mild symptoms. Similar symptoms were prevalent in plants which had been inoculated by sap transmission (experiment IV), consistent with the proposal that reversion or recombination in gene *AC2* to produce wild-type virus had not occurred.

To investigate the viral DNA forms associated with these infections, total nucleic acids were extracted from systemically infected leaves (Etessami *et al.*, 1991) and equal amounts were fractionated by agarose gel electrophoresis in 40 mM-Tris–acetate pH 7·5, 20 mM-sodium acetate, 2 mM-EDTA (TNE buffer). When appropriate, samples were digested with restriction endonucleases in buffers supplied by the manufacturer and subsequently with 5U mungbean nuclease for 30 min at 37 °C to remove viral single-stranded (ss) DNA. Fragment sizes were estimated using BRL 1 kb marker DNA standards. Viral DNAs were detected by Southern hybridization with oligolabelled probes (Feinberg & Vogelstein, 1983) specific for ACMV DNA A [*Dra*I(223)–*Sph*I(2585)], ACMV DNA B [*Pst*I(249)–*Eco*RV(2552)], TGMV DNA A [*Nco*I(345)–*Nco*I(2059)] and TGMV DNA B [*Afl*II(1264)–*Nco*I(1858)], and membranes were washed finally in 0·1 × SSC, 0·1 % SDS at 65 °C to avoid cross-hybridization between genomic components. As is generally the case, more ssDNA than double-stranded, supercoiled (sc) DNA accumulated in tissues infected with wild-type ACMV (AA+AB) and TGMV (TA +TB) although the relative level of ssDNA associated with the TGMV coat protein mutant (TA-AR1-1+TB) was greatly reduced (Fig. 1).

No viral DNAs were detected in symptomless plants inoculated with the ACMV *AC2* mutant (AA-AC2-2+AB) (data not shown), consistent with previous results obtained following the introduction of the mutant

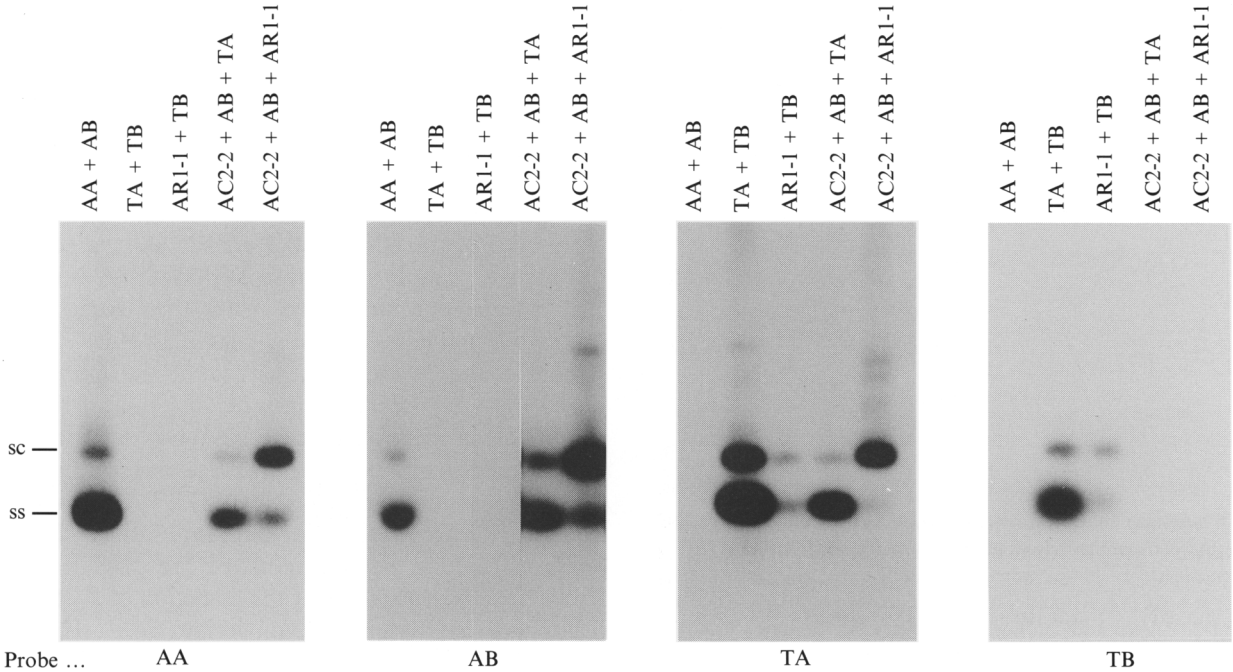

Fig. 1. Southern blot analysis of viral DNAs extracted from *N. benthamiana* co-agroinoculated with ACMV and TGMV genomic components. Plants were co-agroinoculated with combinations of ACMV (AA+AB) and TGMV (TA+TB) genomic components, ACMV *AC2* mutant (AA-AC2-2) and TGMV coat protein mutant (TA-AR1-1). Nucleic acid samples (5 μg) were resolved using an agarose gel containing TNE buffer. Blots were hybridized to probes specific for ACMV DNA A (AA), ACMV DNA B (AB), TGMV DNA A (TA) and TGMV DNA B (TB) and washed under high stringency to avoid cross-hybridization between genomic components. The positions of single-stranded (ss) and supercoiled (sc) DNAs are indicated.

by mechanical inoculation (Etessami *et al.*, 1991). However, when the mutant was co-agroinoculated in the presence of TGMV DNA A (AA-AC2-2+AB+TA), both ACMV genomic components as well as TGMV DNA A were detected in systemically infected symptomatic tissues. When TGMV DNA A was replaced by a coat protein mutant (AA-AC2-2+AB+TA-AR1-1), all three genomic components were detected although the relative level of ssDNA in each case was again reduced.

To ensure that the mutation had been retained in mutant AA-AC2-2 during systemic infection, total nucleic acids extracted from leaves of three plants infected with the clone combinations AA-AC2-2+AB+TA and AA-AC2-2+AB+TA-AR1-1 were digested with *Hpa*I and *Mlu*I (Fig. 2). Wild-type ACMV, containing only a single *Mlu*I site, was linearized under these conditions (lane 1) and the AA-AC2-2 mutant, containing an additional *Hpa*I site, produced fragments of the expected sizes of 1862 and 917 bp (lanes 2 and 3), indicating that the mutation had been retained. A DNA A fragment (nucleotides 1329–1782) encompassing the *AC2* coding sequence was PCR-amplified from total nucleic acids extracted from a plant infected with the clone combination AA-AC2-2+AB+TA essentially as described (Stanley, 1995). Direct sequence analysis of the fragment using component-specific primers confirmed that the mutation had been retained and indicated that no additional compensating mutation had been introduced within the coding sequence.

Previous investigations have indicated that agroinoculation facilitates the amplification of DNA A alone in systemically infected tissues (Klinkenberg & Stanley, 1990; Evans & Jeske, 1993). To eliminate the possibility that agroinoculation contributes significantly to the infectivity of the mixed components, an attempt was made to transmit the progeny by mechanical means. Virus from systemically infected leaves was sap transmissible at low frequency (Table 1, experiment IV) and induced mild symptoms similar to those produced after agroinoculation. DNA extracted from systemically infected leaves of such plants gave a similar hybridization profile to those shown in Fig. 1, and restriction analysis using *Hpa*I and *Mlu*I once again demonstrated that the *AC2* mutation had been retained (data not shown). The ability to sap transmit the mixture of genomic components indicates that the use of agroinoculation to introduce the cloned components into plants does not contribute significantly to the complementation phenomenon. Interestingly, dot blot analysis of plants that remained asymptomatic after sap transmission revealed the presence of TGMV DNA A alone in the upper leaves

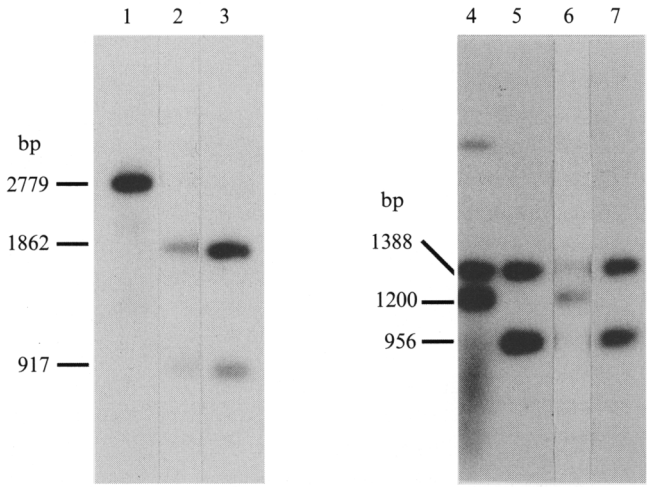

Fig. 2. Restriction endonuclease analysis of ACMV and TGMV DNA A genomic components extracted from systemically infected *N. benthamiana* leaves. Plants were co-agroinoculated with AA and AB (lane 1); AA-AC2-2, AB and TA (lanes 2 and 6); AA-AC2-2, AB and TA-AR1-1 (lanes 3 and 7); TA and TB (lane 4); TA-AR1-1 and TB (lane 5). Samples in lanes 1–3 were digested with *Hpa*I and *Mlu*I and probed for ACMV DNA A and those in lanes 4–7 were digested with *Bam*HI and *Pvu*I and probed for TGMV DNA A. Fragment sizes are indicated.

of two of the five plants examined. Presumably, on these occasions, ACMV DNA B gene products had facilitated the systemic spread and amplification of the TGMV component while the ACMV components themselves remained below the level of detection in, or had not reached, these tissues.

Wild-type TGMV DNA A, containing unique *Bam*HI and *Pvu*I sites, produced the expected fragments of 1388 and 1200 bp when total nucleic acid extracts of plants agroinoculated with clone combinations TA+TB and AA-AC2-2+AB+TA were digested with these enzymes (Fig. 2, lanes 4 and 6). The loss of the 1200 bp fragment and its replacement by a 956 bp fragment verified the presence of the additional *Pvu*I site associated with the coat protein mutation within TGMV DNA A in extracts of plants agroinoculated with the clone combinations TA-AR1-1+TB and AA-AC2-2+AB+TA-AR1-1 (lanes 5 and 7). Digestion with *Xho*I and *Bam*HI linearized the viral DNA, confirming the loss of the unique *Xho*I site in the progeny of TA-AR1-1 (data not shown).

Proteins were extracted from leaves displaying systemic symptoms, resolved by SDS–PAGE and transferred to nitrocellulose for immunoblotting. The supernatant fraction S1 was used for the detection of coat protein and extract E2 for the detection of BV1 and BC1 as previously described (von Arnim *et al.*, 1993). Blots were probed using polyclonal antisera raised against purified ACMV (Townsend *et al.*, 1985) or synthetic oligopeptides specific to BV1 and BC1 (von Arnim *et al.*, 1993), and developed using phosphatase-conjugated goat anti-rabbit IgG (Sigma) as described (Blake *et al.*, 1984). ACMV coat protein was detected in plants inoculated with wild-type ACMV (AA+AB) and in plants co-agroinoculated with the clone combination AA-AC2-2+AB+TA (Fig. 3*a*), implying that transactivation of ACMV coat protein expression occurs in the presence of complementing AL2. The band detected in extracts from

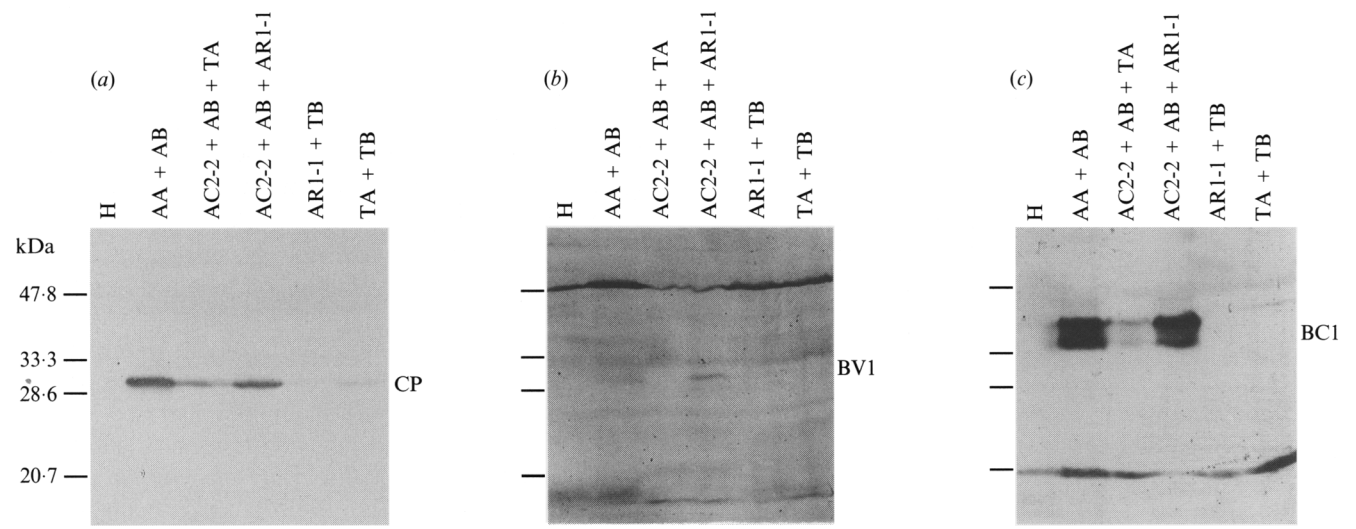

Fig. 3. Western blot analysis of ACMV-encoded proteins extracted from systemically infected *N. benthamiana* leaves. Proteins were extracted from either a healthy plant (H) or plants co-agroinoculated with combinations of ACMV (AA+AB) and TGMV (TA+TB) genomic components, ACMV *AC2* mutant (AA-AC2-2) and TGMV coat protein mutant (TA-AR1-1). Blots were probed using polyclonal antiserum raised against purified ACMV (*a*) and synthetic oligopeptides specific to ACMV BV1 (*b*) and BC1 (*c*). The positions of coat protein (CP), BV1 and BC1 are indicated. Prestained markers (Bio-Rad) were used as molecular mass standards (kDa).

plants infected with TGMV (TA+TB), which has a slightly faster migration than that of ACMV coat protein, probably corresponds to TGMV coat protein that is known to be serologically related to its ACMV counterpart (Stein *et al.*, 1983). However, coat protein was also detected in plants co-agroinoculated with the clone combination that included the TGMV coat protein mutant (AA-AC2-2+AB+TA-AR1-1), ensuring that antiserum was detecting ACMV rather than TGMV coat protein in this instance and confirming that AL2-mediated transactivation of ACMV coat protein expression had occurred. An extremely faint band comigrating with ACMV coat protein, associated with the TGMV coat protein mutant (TA-AR1-1+TB), was not visible on other blots and may have arisen as a result of slight contamination from the adjacent lane on this occasion.

Because both ACMV *BV1* and *BC1* are essential for infectivity and symptom development (Etessami *et al.*, 1988), it is reasonable to assume that AL2 additionally transactivates one or both of these genes during the mixed infection, as suggested from earlier work (Sunter *et al.*, 1990; Sunter & Bisaro, 1991, 1992; Gröning *et al.*, 1994; Haley *et al.*, 1992). The presence of both DNA B gene products was confirmed in plants infected with the clone combination AA-AC2-2+AB+TA-AR1-1 (Fig. 3*b*, *c*). As previously reported (von Arnim *et al.*, 1993), BV1 migrated as a single band and BC1 as multiple bands during SDS–PAGE. BC1 was also detected in plants co-agroinoculated with the clone combination AA-AC2-2+AB+TA, albeit at reduced levels, although BV1 was not detected in the same extract. Coat protein was also expressed at a reduced level (Fig. 3*a*) and correlated with the low levels of scDNA found in these tissues (Fig. 1). In contrast, co-infection with the TGMV coat protein mutant resulted in a reduction in the relative abundance of TGMV ssDNA and concomitant increase in scDNA, offering an explanation for the presence of relatively high levels of the three ACMV proteins in the systemically infected tissues that were sampled.

A reduction in the level of ssDNA associated with coat protein mutants in *N. benthamiana* has previously been described for both ACMV and TGMV (Stanley & Townsend, 1986; Elmer & Rogers, 1990). Interestingly, ssDNA accumulates in tissues infected with the ACMV *AC2* mutant in the presence of wild-type TGMV DNA A (Fig. 1, clone combination AA-AC2-2+AB+TA) but not in the presence of the TGMV coat protein mutant (clone combination AA-AC2-2+AB+TA-AR1-1). The accumulation of ssDNA in wild-type infections has been attributed to the availability of coat protein to encapsidate it, making it unavailable for re-entry into the replication cycle (Stanley & Townsend, 1986). On this basis, however, it is not clear why reduced levels of ssDNA are found in plants co-infected with ACMV *AC2* mutant and TGMV coat protein mutant even though these tissues contain reasonable quantities of ACMV coat protein. One possibility is that virus particle formation by intact ACMV coat protein may be disrupted in the presence of the amino-terminal portion of the defective coat protein encoded by the TGMV mutant.

The ability of ACMV to mediate the systemic infection of *N. benthamiana* by TGMV DNA A following co-agroinoculation of genomic components (Frischmuth *et al.*, 1993) has allowed us to test *in planta* whether TGMV gene *AL2* can functionally complement an ACMV *AC2* mutant. The ACMV mutant is unable to systemically infect this host when co-inoculated with DNA B (Etessami *et al.*, 1991). By screening for the production of systemic symptoms, we have demonstrated that the ACMV *AC2* mutant can systemically infect *N. benthamiana* in the presence of co-agroinoculated DNA B and TGMV DNA A, showing that AL2 has the ability to fully complement defective AC2 expression albeit at low frequency. Recently, ACMV AC2 has been shown to transactivate GUS expression from the TGMV coat protein and BR1 promoters in tobacco protoplasts (Sunter *et al.*, 1994). Our observation using the reciprocal combination (i.e. AL2 complementation of AC2 function) serves to emphasize the functional homology of these two proteins and additionally demonstrates that complementation extends to the infection of whole plants. Such reciprocal compatibility of gene function does not always occur; previous work on these particular viruses has shown that although ACMV DNA B genes facilitate the spread of TGMV, TGMV DNA B genes are not only unable to spread ACMV but also actively inhibit this process (von Arnim & Stanley, 1992*b*; Frischmuth *et al.*, 1993).

Having demonstrated that ACMV-mediated spread of TGMV DNA A occurs efficiently and at high frequency (Frischmuth *et al.*, 1993) we consider it unlikely that the requirement for co-infection of three genomic components is solely responsible for the low level of infectivity and mild symptoms associated with the complemented ACMV *AC2* mutant. Hence, our results suggest that AL2 transactivates expression of coat protein and one or both of the essential ACMV DNA B genes with reduced efficiency compared with AC2. This is consistent with the observed decrease in the level of transactivation of TGMV *BR1* by AC2 in tobacco protoplasts (Sunter *et al.*, 1994) and probably reflects the divergent nature of Old World and New World geminiviruses (Padidam *et al.*, 1995), represented by ACMV and TGMV, respectively.

ACMV differs from TGMV in that it has a putative gene (*AV2*) of unknown function that overlaps the 5′

terminus of the coat protein gene. The most abundant virion-sense transcript initiates immediately upstream of *AV2* suggesting that it might function as a polycistronic message for both AV2 and coat protein expression (Townsend *et al.*, 1985). Although ACMV *AC2* and TGMV *AL2* have been shown to be functionally interchangeable, it remains to be seen if AC2 specifically transactivates expression of coat protein, as has been demonstrated for AL2, or if it has an additional effect on AV2 expression. The control of ACMV DNA A virion-sense gene expression is the subject of a current investigation.

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